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Activation of Influenza A Viruses by Host Proteases from Swine Airway Epithelium

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Pigs are important natural hosts of influenza A viruses, and due to their susceptibility to swine, avian, and human viruses, they may serve as intermediate hosts supporting adaptation and genetic reassortment. Cleavage of the influenza virus surface glycoprotein hemagglutinin (HA) by host cell proteases is essential for viral infectivity. Most influenza viruses, including human and swine viruses, are activated at a monobasic HA cleavage site, and we previously identified TMPRSS2 and HAT to be relevant proteases present in human airways. We investigated the proteolytic activation of influenza viruses in primary porcine tracheal and bronchial epithelial cells (PTEC and PBEC, respectively). Human H1N1 and H3N2 viruses replicated efficiently in PTECs and PBECs, and viruses containing cleaved HA were released from infected cells. Moreover, the cells supported the proteolytic activation of HA at the stage of entry. We found that swine proteases homologous to TMPRSS2 and HAT, designated swTMPRSS2 and swAT, respectively, were expressed in several parts of the porcine respiratory tract. Both proteases cloned from primary PBECs were shown to activate HA with a monobasic cleavage site upon coexpression and support multicycle replication of influenza viruses. swAT was predominantly localized at the plasma membrane, where it was present as an active protease that mediated activation of incoming virus. In contrast, swTMPRSS2 accumulated in the trans-Golgi network, suggesting that it cleaves HA in this compartment. In conclusion, our data show that HA activation in porcine airways may occur by similar proteases and at similar stages of the viral life cycle as in human airways.

Influenza A viruses circulate in a wide range of avian and mammalian hosts, including poultry, pigs, and humans, posing a serious threat to both animal and human health. Human influenza A viruses are a major cause of acute infection of the respiratory tract that affects millions of people during seasonal epidemics and occasional pandemics. Avian and swine influenza A viruses are responsible for outbreaks in poultry farms and pig herds, respectively, causing substantial morbidity and great economic losses as well as the permanent risk of cross-species transmission of new viruses to humans.

Influenza A viruses belong to the family of *Orthomyxoviridae* and have a single-stranded, negative-sense RNA genome which consists of eight segments encoding up to 15 proteins (1, 2). The virion possesses a lipid envelope that contains the two major spike glycoproteins, hemagglutinin (HA) and neuraminidase (NA). On the basis of antigenic criteria for HA and NA, influenza A viruses are currently divided into 17 HA (H1 to H17) and 10 NA (N1 to N10) subtypes (3).

Pigs are important mammalian hosts of influenza A viruses. The predominant subtypes currently found in swine, H1N1, H1N2, and H3N2, circulate among pig herds throughout the year and are enzootic in swine populations worldwide. Importantly, pigs are susceptible to infection with avian and human influenza viruses due to the presence of both *N*-acetylneuraminic acids linked to galactose by an α2,3 linkage (avian type) and α2,6 linkage (human type) in their respiratory tract (4). In 1979, H1N1 influenza viruses possessing gene segments of avian viruses were isolated from pigs, demonstrating reassortment of avian and mammalian influenza viruses in swine (5, 6). Thus, pigs were recognized as intermediate hosts and considered mixing vessels that play an important role in the emergence of new influenza viruses with pandemic potential (7–11).

Influenza virus infection is initiated by HA, which binds to the cell surface receptors and mediates fusion of the viral envelope with the endosomal membrane. HA is synthesized as precursor protein HA0, which must be cleaved by a host cell protease into the HA1 and HA2 subunits to gain its fusion capacity. Cleavage of HA exposes the hydrophobic fusion peptide at the N terminus of HA2 and is a prerequisite for conformational changes in the endosome at low pH that trigger membrane fusion. Therefore, HA cleavage is essential for virus infectivity and spread (12, 13). Most influenza viruses, including human and swine viruses of subtypes H1, H2, and H3, contain a single arginine (R) at the HA0 cleavage site and are activated by trypsin *in vitro* (14). Appropriate trypsin-like proteases are present in a limited number of tissues, such as the respiratory or the intestinal tract, hence limiting virus spread to these tissues. Relevant human HA-activating proteases were unknown for a long time. In 2006, we identified the type II transmembrane proteases TMPRSS2 (transmembrane protease serine S1 member 2) and HAT (human airway trypsin-like protease) as proteases that cleave HA with a monobasic cleavage site in the human airway epithelium (15). By use of Madin-Darby canine kidney (MDCK) cells with doxycycline-inducible expression of each protease, it has been shown that activation of HA occurs by membrane-bound forms of the proteases and takes place in different cellular compartments and at different stages during the viral life cycle (16, 17). Cleavage of HA by HAT was demonstrated to occur on the cell surface, either during assembly and budding of new virions or late in infection during attachment and entry into a

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new cell. In contrast, HA activation by TMPRSS2 is accomplished within the cell, during its transport to the cell surface.

Primary porcine airway epithelial cell cultures turned out to provide suitable model systems to investigate infection and replication of influenza viruses of avian, human, or swine origin (18–25). Recent studies demonstrated that HA with a monobasic cleavage site is activated in these cultures without the need for exogenous trypsin (18, 21, 25), but the relevant proteases have so far not been identified or characterized in more detail.

In this study, we used primary porcine tracheal epithelial cells (PTECs) and porcine bronchial epithelial cells (PBECs) to identify HA-activating proteases in the respiratory tract of swine. We show that primary PTECs and PBECs support multicycle replication of human H1N1 and H3N2 influenza viruses due to the proteolytic activation of HA either prior to release of progeny virus or upon entry into the cells. Moreover, we demonstrate that proteases homologous to TMPRSS2 and HAT, designated swine TMPRSS2 (swTMPRSS2) and swine airway trypsin-like protease (swAT), respectively, are expressed in these cells and are able to activate influenza A viruses with a monobasic cleavage site. Immunofluorescence analysis and studies on the enzymatic activity of swTMPRSS2 and swAT revealed that the cleavage of HA may occur by similar proteases and at similar stages during the viral life cycle in airway epithelial cells of swine and human.

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MATERIALS AND METHODS

Isolation of primary airway epithelial cells from swine. Primary porcine airway epithelial cells were obtained from the tracheas and bronchi of 4- to 6-month-old pigs from a butchery. Preparation of respiratory epithelial cells was based on modified isolation protocols previously described (26, 27). Distal trachea and proximal bronchi were excised from surrounding soft tissues and lung parenchyma, dissected, washed with phosphate-buffered saline (PBS), and incubated in Dulbecco's modified Eagle medium (DMEM; Gibco) containing 100 U/ml penicillin, 100 μg/ml streptomycin, 200 mM glutamine, 2.5 μg/ml amphotericin (Invitrogen), 50 μg/ml kanamycin (Gibco), 10 μg/ml enrofloxacin (Sigma), 1 μg/ml clotrimazole (Sigma), and 0.5 mg/ml dithiothreitol (DTT; Sigma) for 24 h at 4°C. Tracheal and bronchial specimens were rinsed with PBS and digested with 0.1% protease type XIV (Sigma) in DMEM (composed as described above) for 20 to 24 h at 4°C. Afterwards, porcine tracheal and bronchial specimens were washed with PBS and longitudinally sliced. Epithelial cells were harvested by scraping the luminal surface with a scalpel, collected in PBS, and centrifuged at 1,000 rpm for 10 min at room temperature. Cells were resuspended in porcine airway epithelial cell growth medium (PAEGM; consisting of airway epithelial cell growth medium [AEGM; ready to use; Promocell] supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 200 mM glutamine, 2.5 μg/ml amphotericin), filtered through a 250-μm-pore-size polyester monolen mesh filter (neoLab), and seeded into type I collagen (BD Biosciences)-coated cell culture flasks. Tracheal and bronchial tissue specimens were processed individually, and the respective epithelial cells were pooled before seeding. Within 3 to 5 days at 37°C in a 5% CO_2 atmosphere, cultures of primary nondifferentiated PTECs and PBECs reached confluence and were applicable for experiments or cryopreservation. Primary porcine epiglottic epithelial cells (PEECs) were obtained and cultivated similarly. The morphology of the PTEC and PBEC monolayers was examined by light microscopy (Nikon Eclipse TS 100 microscope), and the epithelial origin was analyzed by immunofluorescence staining of cytokeratin (epithelial marker protein) and E-cadherin (adherens junction marker), as described below.

Cell lines, viruses, and plasmids. Human embryonic kidney (293T) cells, human hepatocyte-derived carcinoma (Huh7) cells, and MDCK cells were cultivated in DMEM supplemented with 10% fetal calf serum (FCS; Gibco), glutamine, penicillin, and streptomycin at 37°C in a 5% CO_2 atmosphere. Human influenza viruses A/Memphis/14/96 (H1N1) [A/Memphis (H1N1); provided by Robert Webster, St. Jude Children's Research Hospital, Memphis, TN], 2009 pandemic virus A/Hamburg/5/09 (H1N1) [A/Hamburg (H1N1)pdm09; provided by Mikhail Matrosovich, Institute of Virology, Philipps University Marburg], and A/Aichi/2/68 (H3N2) [A/Aichi (H3N2)] were used in this study. Viruses were propagated in MDCK cells in infection medium (DMEM supplemented with 0.1% bovine serum albumin, glutamine, and antibiotics) containing 1 μg/ml tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (Sigma). Cell supernatants were cleared by low-speed centrifugation and stored at −80°C. Influenza virus A/Memphis/14/96 (H1N1) containing noncleaved HA0 was generated by infection of MDCK cells in the absence of trypsin, as described elsewhere (17).

The mammalian expression plasmids pCAGGS-HAT and pCAGGS-TMPRSS2, coding for HAT and TMPRSS2, respectively, with a C-terminal FLAG epitope as well as pCAGGS-H1 encoding HA of A/Hamburg/5/09 (H1N1)pdm09 and the expression plasmid pSG(5):bfur-furin encoding bovine furin have been described previously (15, 28, 29).

RNA isolation and RT-PCR analysis. Total RNA was isolated from either primary PEECs, PTECs, and PBECs or porcine lung tissue using an RNeasy minikit (Qiagen). Detection of swTMPRSS2- and swAT-specific mRNA was assessed by reverse transcription-PCR (RT-PCR), carried out with 2 μg of total RNA using a One-Step RT-PCR kit (Qiagen) and protease-specific primers that amplify nucleotides (nt) 613 to 1821 of swAT mRNA and nt 1 to 1488 of swTMPRSS2 mRNA (primers swAT-ATG-for [5′-C ATG CCA GCA ACA GTA CG-3′] and swAT-rev [5′-CTA GAT CCC AGT TTG TTG-3′] and primers swTPMRSS2-EcoRI-for [5′-C GAA TTC GGA ATG GCT TTA AAC TCA G-3′] and swTMPRSS2-NotI-rev [5′-A TGC GGC CGC TCC TTA GCT GTT TGC CCT CAT-3′]). For detection of α-tubulin mRNAs, primers specific for swine α-tubulin were used (primers α-tub-for [5′-TCA GTG CGT TAC TCA CCT CG-3′] and α-tub-rev [5′-AAT CAG AGT GCT CCA GGG TG-3′]). RT-PCR products were analyzed by electrophoresis on a 0.8% agarose gel, stained with ethidium bromide, and subsequently sequenced to confirm the identity of the amplified RT-PCR products.

Cloning of swine proteases swTMPRSS2 and swAT. The cDNAs encoding swTMPRSS2 (GenBank accession number BAF76737.1) or swAT (predicted sequence; GenBank accession number XM_003129055.3) were cloned from total RNA of primary PBECs by RT-PCR using primers specific for swTMPRSS2 or swAT, as described above (primers swTPMRSS2-EcoRI-for [5′-C GAA TTC GGA ATG GCT TTA AAC TCA G-3′] and swTMPRSS2-NotI-FLAG-rev [5′-A TGC GGC CGC TTA CTT ATC GTC ATC GTC CTT ATA GTC GCT GTT TGC CCT CAT-3′] and primers swAT-EcoRI-ATG-for [5′-C GAA TTC GGA ATG CCA GCA ACA GTA C-3′] and swAT-NotI-FLAG-rev [5′-A TGC GGC CGC CTA CTT ATC GTC ATC GTC CTT ATA GTC GAT CCC AGT TTG-3′]). The amplified PCR products encoding swTMPRSS2 or swAT, each with a C-terminal FLAG epitope sequence, were subcloned into the expression plasmid pCAGGS using EcoRI and NotI restriction sites.

Transfection of cells and transient protein expression. For transient protein expression, 293T cells were transfected with pCAGGS-swTMPRSS2-FLAG, pCAGGS-swAT-FLAG, pCAGGS-TMPRSS2-FLAG, or pCAGGS-HAT-FLAG using Lipofectamine 2000 (Invitrogen) and incubated for 24 h. Subsequently, cell lysates were subjected to SDS-PAGE under reducing conditions and analyzed by Western blotting using polyclonal anti-FLAG serum (Sigma), as described below. To analyze the coexpression of HA with swTMPRSS2 or swAT, 293T cells were seeded in 12-well plates and cotransfected with pCAGGS-H1 and either pCAGGS-swTMPRSS2 or pCAGGS-swAT for 48 h. As a control, HA-expressing cells were treated with 10 μg/ml TPCK-treated trypsin in PBS for 30 min

at 37°C at 48 h posttransfection (p.t.). Cell lysates were analyzed by Western blotting using rabbit serum against HA.

For immunofluorescence analysis of protease expression, MDCK or Huh7 cells grown on coverslips were transfected with plasmids encoding either swTMPRSS2, swAT, or furin. At 24 h p.t., cells were analyzed by fluorescence microscopy, as described below.

SDS-PAGE and Western blot analysis. Cells were washed with PBS, resuspended in reducing SDS sample buffer, and lysed by sonication and heating at 95°C for 5 min. Proteins were subjected to SDS-PAGE (12% polyacrylamide gel) and transferred to a polyvinylidene difluoride (PVDF) membrane (GE Healthcare). Subsequently, proteins were detected by incubation with the respective primary antibodies and species-specific horseradish peroxidase (HRP)-conjugated secondary antibodies (Dako), followed by incubation with enhanced chemiluminescence peroxidase substrate (Pierce) and exposure of the membrane to autoradiography films (CEA).

Virus-containing supernatants were cleared from the cell debris by low-speed centrifugation (6,000 rpm, 5 min), and virus was pelleted by ultracentrifugation (28,000 rpm, 2 h, 4°C). Virus pellets were resuspended in reducing SDS sample buffer, heated at 95°C for 5 min, and subjected to SDS-PAGE and Western blot analysis with HA-specific antibodies. Polyclonal rabbit serum against H1 was provided by Mikhail Matrosovich, Institute of Virology, Philipps University Marburg; polyclonal serum against H3 was derived from rabbits immunized with A/Aichi/2/68 (H3N2).

Infection of cells and multicycle virus replication. For analysis of infection and multicycle replication of influenza viruses in primary PTECs and PBECs as well as transient swTMPRSS2- or swAT-expressing MDCK cells, cell monolayers were infected with virus at a multiplicity of infection (MOI) of 0.01 to 0.001 in PAEGM or infection medium and incubated for 24 h at 37°C. Subsequently, infected cells were immunostained against the viral nucleoprotein (NP) as described previously (15). Briefly, cells were fixed with 4% paraformaldehyde (PFA; Roth) in PBS and permeabilized with 0.3% Triton X-100 (Sigma) in PBS. Infected cells were immunostained using a NP-specific antibody and a species-specific HRP-conjugated secondary antibody, followed by incubation with the peroxidase substrate TrueBlue (KPL). Representative images were recorded using a Nikon Eclipse TS 100 microscope. To analyze growth kinetics in PTECs and PBECs, primary cell cultures were inoculated with virus at a low MOI of 0.001 to 0.0001 in PAEGM for 1 h at 37°C. Subsequently, cells were washed with PBS and incubated with fresh PAEGM for 72 h. At 16, 24, 48, and 72 h postinfection (p.i.), samples of PTEC and PBEC supernatants were treated with 1 μg/ml TPCK-treated trypsin for 1 h at 37°C or remained untreated, and virus titers were determined by plaque assay with Avicel overlay, as described previously (30).

Immunofluorescence analysis and subcellular localization studies. For detection of intracellular proteins, cells were fixed and permeabilized with methanol-acetone (1:1) and blocked by incubation with 2% bovine serum albumin (Sigma). Afterwards, cells were subjected to immunostaining by sequential incubation with the respective primary antibodies and secondary fluorescence-labeled antibodies, each for 1 h at room temperature in a humid chamber. Cell nuclei were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI; Sigma). Subsequently, cells were rinsed with PBS and mounted on microscope slides using the Mowiol reagent (Calbiochem). Representative images were recorded using a Zeiss Axiovert 200 M microscope.

Cytokeratins were stained with a combination of monoclonal antibodies for basic and acidic cytokeratins (Acris) and species-specific fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (Dako). E-cadherin was stained with an anti-E-cadherin antibody (BD Biosciences) and tetramethylrhodamine isothiocyanate (TRITC)-labeled secondary antibodies (Dako). To visualize transiently expressed swTMPRSS2 and swAT, a polyclonal FLAG antiserum (Sigma) and FITC-conjugated secondary antibodies (Dako) were used. Coexpressed swTMPRSS2 and swAT were detected using polyclonal FLAG and HAT antisera (Genovac GmbH), respectively, and species-specific FITC- and TRITC-conjugated secondary antibodies. Endogenous TGN38 was detected with a monoclonal TGN38 antibody (Transduction Laboratories), followed by Alexa Fluor 568-labeled secondary antibodies. Transiently expressed furin was stained using a furin-specific antiserum (31) and Alexa Fluor 568-conjugated secondary antibodies (Invitrogen). Endogenous swTMPRSS2 and swAT were detected with a polyclonal anti-TMPRSS2 or anti-HAT serum (Genovac GmbH), respectively, and appropriate FITC-labeled secondary antibodies (Sigma-Aldrich, Dako).

Cell surface immunofluorescence analysis. MDCK cells transiently expressing protease were incubated with polyclonal anti-FLAG serum for 1 h at 4°C prior to fixation with 2% PFA. Primary antibodies were visualized by use of species-specific FITC-labeled secondary antibodies. Cell surface expression of endogenous swTMPRSS2 and swAT was analyzed with a polyclonal anti-TMPRSS2 or anti-HAT serum, respectively, and species-specific FITC-conjugated secondary antibodies.

Protease activity at the cell surface. To examine the enzymatic activity of swTMPRSS2 and swAT at the cell surface, MDCK cells were grown in 96-well plates and transfected with either pCAGGS-swTMPRSS2 or pCAGGS-swAT, respectively. At 24 h p.t., cells were washed with PBS and protease activity was assayed by incubation of cells with the fluorogenic peptide substrate Boc–Gly–Pro–Arg–7-amino-4-methylcumarin (AMC) (Bachem) at a final concentration of 25 μM in DMEM for 45, 90, and 150 min at 37°C in 5% CO_2, as described previously (17). Hydrolysis of the peptide was monitored from the fluorescence intensity of free AMC using a Perkin-Elmer LS 55 luminescence spectrometer at excitation and emission wavelengths set at 350 nm and 460 nm, respectively. For each sample, activity was measured in triplicate and measured in relative fluorescence units (RFU).

Infection assay with virions containing HA0. Monolayers of PTECs and PBECs or MDCK cells transiently expressing protease were washed with PBS and incubated with 50 to 100 μl of noninfectious virus A/Memphis (H1N1) containing noncleaved HA0 at an MOI of 0.1 in infection medium or PAEGM. Subsequently, cells were incubated for 30 min on ice to allow adsorption of virions, shifted to 37°C in a 5% CO_2 atmosphere, and incubated for 10 h to allow a single cycle of replication. Inoculation of cells with virions in the presence of the protease inhibitor aprotinin (50 to 100 μM; AppliChem) served as a control. Subsequently, cells were fixed and immunostained against NP as described above. For each sample, the number of infected cells per well was determined in triplicate.

RESULTS

Proteolytic activation of influenza A viruses by endogenous proteases in primary PTECs and PBECs. In order to study the proteolytic activation of influenza viruses containing a monobasic HA cleavage site in swine airway epithelial cells, we established cultures of primary PTECs and PBECs that were characterized by light microscopy and immunofluorescence staining of the epithelial marker protein cytokeratin and the adherens junction protein E-cadherin (Fig. 1). Primary PTECs and PBECs were infected with human influenza virus A/Hamburg (H1N1)pdm09 or A/Aichi (H3N2) and incubated for 24 h. Immunostaining of infected cells showed the typical comet-like spread of infection, providing evidence for proteolytic activation and multicycle replication of both influenza virus strains in PTEC and PBEC monolayers (Fig. 2A). In addition, the growth kinetics of H1N1 and H3N2 viruses were performed in primary PTECs and PBECs. The cells were infected at a low MOI, and the virus titers in the supernatants were determined by plaque titration at 16, 24, 48, and 72 h p.i. As demonstrated in Fig. 2B, A/Hamburg (H1N1)pdm09 and A/Aichi (H3N2) replicated efficiently in PTEC and PBEC cultures; however, they grew to titers 10-fold higher in bronchial epithelial cells than in tracheal epithelial cells. To analyze the HA cleavage of progeny virus released from the cells, virus-containing supernatants were concentrated by ultracentrifugation at 72 h p.i. and

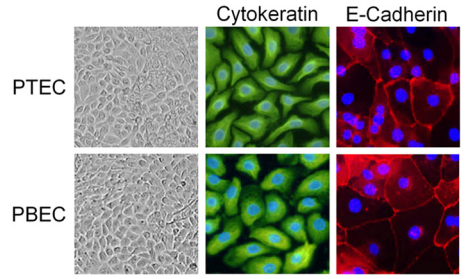

FIG 1 Characterization of isolated PTECs and PBECs. Monolayers of primary PTECs and PBECs were analyzed by light microscopy (magnification, ×10) and immunofluorescence staining of the epithelial marker protein cytokeratin using cytokeratin-specific antibodies and FITC-conjugated secondary antibodies (magnification, ×20). The adherens junction protein E-cadherin was visualized with anti-E-cadherin antibodies and TRITC-conjugated secondary antibodies (magnification, ×40). Nuclei were counterstained with DAPI.

subjected to SDS-PAGE and Western blotting with HA-specific antibodies. Viruses released from primary PTECs and PBECs contained cleaved HA, with an equal efficiency of HA cleavage being found in PTECs and PBECs for both the H1 and H3 subtypes (Fig. 2C). To examine whether exogenous trypsin can increase HA0 cleavage and virus infectivity, virus-containing supernatants were treated with trypsin prior to plaque assay and ultracentrifugation. As shown in Fig. 2B, trypsin treatment revealed no marked increase of virus titers from PBECs and only slightly higher titers in PTEC supernatants, although HA0 was completely cleaved by trypsin (Fig. 2C). These results demonstrate that progeny virus released from primary PTECs and PBECs is already efficiently activated by endogenous proteases. In conclusion, our data reveal efficient multicycle influenza virus replication and HA cleavage in primary PTEC and PBEC cultures, providing evidence for the expression of an appropriate HA-activating protease(s) in tracheal and bronchial epithelial cells from swine.

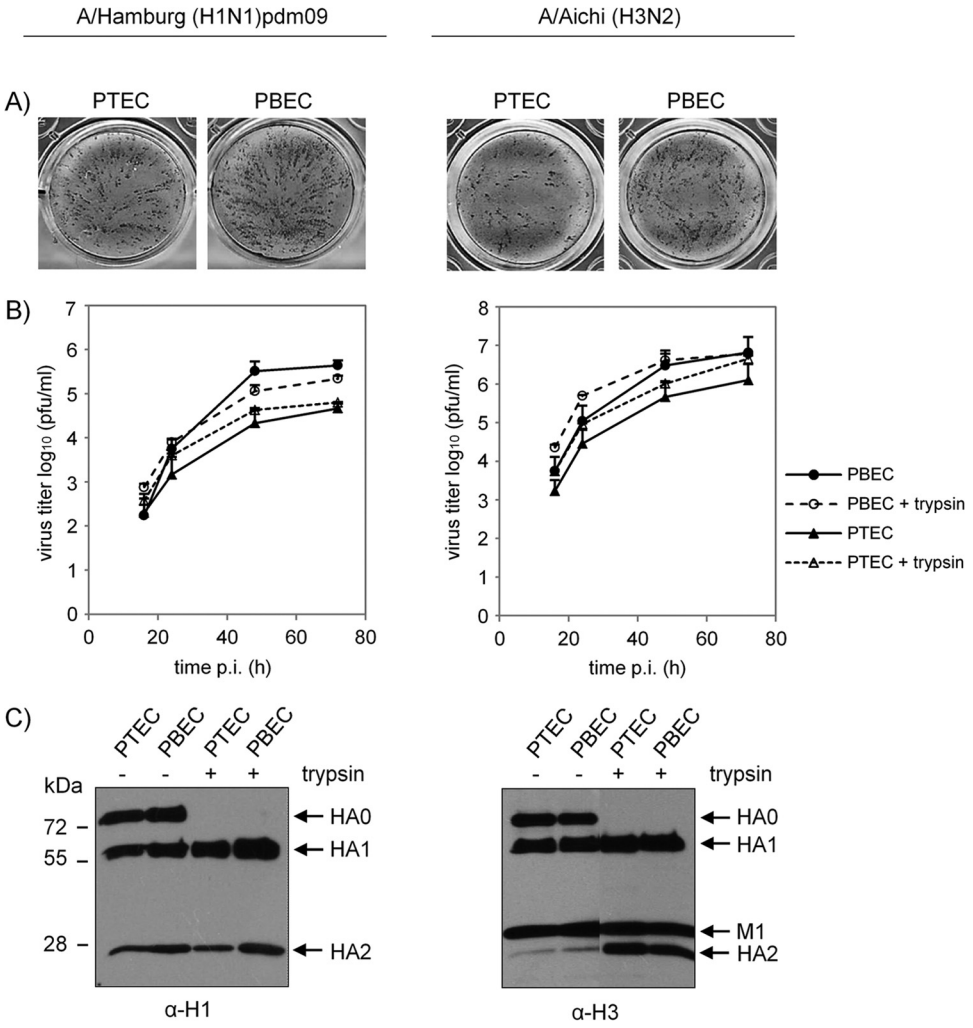

FIG 2 Proteolytic activation and multicycle replication of influenza A viruses in primary porcine airway epithelial cells. (A) Cultures of primary PTECs and PBECs were infected with human influenza virus isolate A/Hamburg (H1N1)pdm09 or A/Aichi (H3N2) at an MOI of 0.003 to 0.007 and incubated for 24 h to allow multiple cycles of virus replication. Infected cells were immunostained against the viral NP. (B) Infection of PTECs and PBECs with A/Hamburg (H1N1)pdm09 (left) or A/Aichi (H3N2) (right) at a low MOI of 0.001 to 0.0001. At the indicated time points, the virus titers of trypsin-treated or untreated supernatants were determined by plaque assay. The results shown are mean values of three independent experiments. (C) At 72 h p.i., virus-containing supernatants were treated with trypsin or remained untreated and pelleted by ultracentrifugation, and HA cleavage was analyzed by SDS-PAGE under reducing conditions and Western blotting using HA-specific antibodies. M1, influenza virus matrix protein.

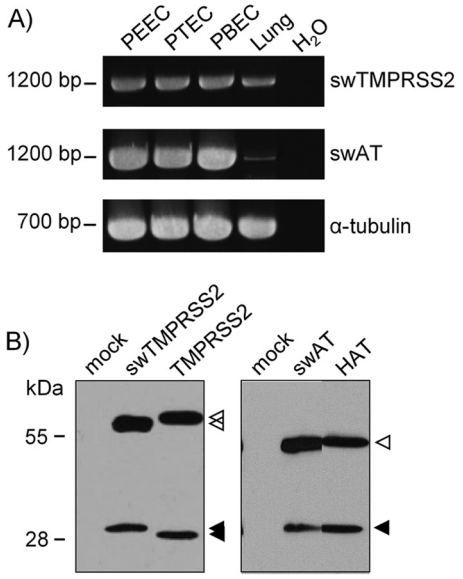

FIG 3 Distribution of swTMPRSS2- and swAT-specific mRNA in respiratory tissues and expression of recombinant swine and human proteases. (A) Total RNA was isolated from PEECs, PTECs, and PBECs as well as porcine lung tissue and used as a target for RT-PCR analysis with a set of primers specific for swTMPRSS2, swAT, or α-tubulin. H_2O was used as a control. (B) 293T cells were transiently transfected with either empty pCAGGS plasmid (mock) or pCAGGS encoding either swTMPRSS2-FLAG, swAT-FLAG, TMPRSS2-FLAG, or HAT-FLAG. At 24 h p.t., cell lysates were subjected to SDS-PAGE under reducing conditions, subsequently transferred to a PVDF membrane, and analyzed by use of FLAG-specific antibodies and HRP-conjugated secondary antibodies. Open and filled arrowheads indicate the zymogen and the mature form of each protease, respectively.

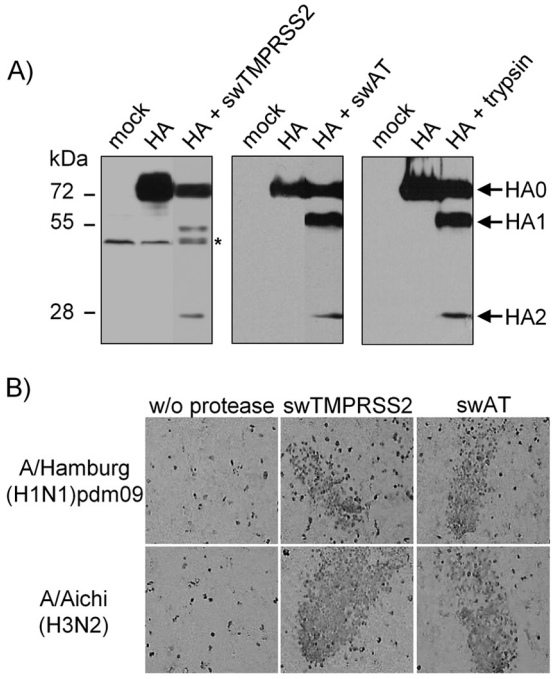

FIG 4 Proteolytic activation of influenza virus HA by swTMPRSS2 and swAT. (A) 293T cells were cotransfected with plasmids encoding HA of A/Hamburg (H1N1)pdm09 and either pCAGGS (mock), pCAGGS-swTMPRSS2, or pCAGGS-swAT. At 48 h p.t., HA cleavage was detected by Western blot analysis of cell lysates using HA-specific antibodies. Treatment of HA-expressing cells with trypsin served as a control. *, a nonspecific band. (B) At 8 h after transfection with empty vector pCAGGS (without [w/o] protease) or protease-encoding plasmids, MDCK cells were infected with influenza virus A/Hamburg (H1N1)pdm09 or A/Aichi (H3N2) at an MOI of 0.01 to 0.001 for 24 h. Infected cells were immunostained against NP. Magnification, ×10.

Proteases swTMPRSS2 and swAT are expressed in different parts of the porcine respiratory tract. Database analysis showed that the swine genome encodes proteases homologous to human HA-activating proteases TMPRSS2 and HAT, here designated swine TMPRSS2 (swTMPRSS2) and swine airway trypsin-like protease (swAT). Amino acid sequence alignments of human and swine proteases revealed 77% homology between swAT and HAT and 81% homology between swTMPRSS2 and TMPRSS2 (data not shown). To examine the expression of swTMPRSS2 and swAT in the airway epithelium of pigs, total RNA was isolated from epiglottic, tracheal, or bronchial epithelial cells and porcine lung tissue and was analyzed by RT-PCR using protease-specific primers. Detection of mRNA specific for α-tubulin was used as a control. PCR products specific for swTMPRSS2 were amplified from all tested airway tissues, with slightly decreased amounts being found in the lung tissue (Fig. 3A). swAT-specific mRNA was found in epithelial cells of the epiglottis, trachea, and bronchi, while only a small amount of swAT mRNA, if any, was detected in the lung tissue. The results indicate that swTMPRSS2 and swAT are expressed in the epiglottis, trachea, and bronchi, whereas only swTMPRSS2 seems to be present in the porcine lung. To analyze whether swTMPRSS2 and swAT may play a role in influenza virus activation, both proteases were cloned from primary PBECs and subcloned into the mammalian expression vector pCAGGS. The recombinant proteases were expressed with a C-terminal FLAG epitope to facilitate protein detection. For expression analysis, 293T cells were transfected with plasmids encoding swTMPRSS2, swAT, or the corresponding human proteases, TMPRSS2 and HAT. Cell lysates were analyzed by SDS-PAGE under reducing conditions and Western blotting using FLAG-specific antibodies. Similar to human proteases, swTMPRSS2 and swAT were found to be expressed as full-length proteins of 60 kDa and 47 kDa, respectively, and as processed forms with an average molecular mass of 30 kDa, probably representing the single-chain zymogen and the C-terminal catalytic domain of the activated two-chain form of each protease. The results suggest that swTMPRSS2 and swAT are expressed as enzymatically active proteases in 293T cells.

Cleavage of HA and multicycle influenza virus replication upon coexpression of swTMPRSS2 and swAT. To investigate if swTMPRSS2 and swAT are able to cleave influenza virus HA with a monobasic cleavage site, each protease was coexpressed with the HA of the 2009 pandemic virus A/Hamburg (H1N1)pdm09 in 293T cells for 48 h. Subsequently, cell lysates were analyzed for HA cleavage by SDS-PAGE and immunoblotting using HA-specific antibodies. As shown in Fig. 4A, coexpression of either swTMPRSS2 or swAT resulted in cleavage of the precursor protein HA0 (75 kDa) into the subunits HA1 (55 kDa) and HA2 (25 kDa), similar to treatment of HA-expressing cells with trypsin. As expected, no cleavage was observed in cells expressing only HA due to the lack of an appropriate HA-activating protease in 293T cells. To examine whether HA cleavage by swTMPRSS2 and swAT supports proteolytic activation of HA and virus spread, MDCK cells were transfected with protease expression plasmids and subsequently infected with A/Hamburg (H1N1)pdm09 or A/Aichi (H3N2) for

24 h. Immunostaining of infected cells demonstrated multicycle replication and spread of both virus strains in cells expressing swTMPRSS2 or swAT (Fig. 4B). In contrast, only initially infected cells were visible in cells without expression of any HA-activating protease. Taken together, these data demonstrate that recombinant swTMPRSS2 and swAT are expressed as enzymatically active proteases which are able to activate influenza A viruses with a monobasic cleavage site.

Expression of swTMPRSS2 and swAT differs in subcellular localization. The present data strongly suggest that swTMPRSS2 and swAT support activation of influenza virus HA in the respiratory tract of pigs. In order to characterize both proteases in more detail, their subcellular localization was examined. On the basis of their predicted domain structure, swTMPRSS2 and swAT belong to the type II transmembrane proteases (TTSPs), a family of proteases described to be expressed on the cell surface (32). To determine cell surface expression of swTMPRSS2 and swAT, the proteases were transient expressed in MDCK cells for 24 h and subsequently monitored by indirect immunofluorescence staining with FLAG-specific antibodies and FITC-conjugated secondary antibodies. As shown in Fig. 5A, both swTMPRSS2 and swAT were found to be expressed at the plasma membrane. Interestingly, immunofluorescence microscopy of swTMPRSS2 and swAT in permeabilized MDCK cells revealed different patterns of subcellular distribution. While swAT was mainly localized at the plasma membrane, swTMPRSS2 accumulated within the cell (Fig. 5B). In addition, swTMPRSS2 and swAT were transiently coexpressed in Huh7 cells, and immunofluorescence analysis proved the distinct subcellular localization of both enzymes (Fig. 5C). To further specify the intracellular distribution of swTMPRSS2, colocalization studies were performed with the trans-Golgi network (TGN)-residing proteins TGN38 and furin (29, 33). For this purpose, Huh7 cells were transfected with pCAGGS-swTMPRRS2-FLAG for 24 h, fixed, permeabilized, and immunostained against swTMPRSS2 and endogenous TGN38 (Fig. 5D). Although furin is endogenously expressed in Huh7 cells as well, the expression levels of the protease were too low to be detected by use of the furin-specific antibody. Therefore, swTMPRSS2 and furin were transiently coexpressed in Huh7 cells and visualized using FLAG- and furin-specific antibodies, respectively, and fluorescence-labeled secondary antibodies (Fig. 5E). As demonstrated in Fig. 5D and E, swTMPRSS2 colocalized with both TGN38 and furin. In contrast, only partial colocalization of swAT and furin in perinuclear vesicle-like structures was observed in Huh7 cells, and swAT was predominantly localized at the plasma membrane (Fig. 5F). In addition, the subcellular localization of endogenous swTMPRSS2 and swAT was analyzed in primary PTEC and PBEC cultures. As shown in Fig. 5G, swTMPRSS2 and swAT were detected at the plasma membrane of primary PTECs and PBECs. Expression of endogenous swTMPRSS2 at the cell surface appeared to be less distinctive, and the protease seemed to accumulate within the cell, whereas prominent surface staining was found for swAT. In permeabilized PTECs and PBECs, swTMPRSS2 revealed a perinuclear, dot-like vesicular distribution (Fig. 5H). Endogenous swAT was mainly localized at the plasma membrane, although some perinuclear and cytoplasmic staining was detected as well (Fig. 5H). Taken together, the results show that swAT is predominantly expressed at the plasma membrane, whereas swTMPRSS2 is present at the cell surface as well but accumulates in the TGN. In addition, immunofluorescence staining of swAT and swTMPRSS2 in primary PTECs and PBECs provides evidence for expression of both proteases in epithelial cells of the trachea and bronchi of swine.

Protease activity at the cell surface and proteolytic activation of incoming viruses. Human proteases TMPRSS2 and HAT were shown to be expressed at the plasma membrane; however, only HAT was found to exhibit enzymatic activity on the cell surface (17). To assay the enzymatic activity of swTMPRSS2 and swAT on the cell surface, transient protease-expressing MDCK cells were incubated with the fluorogenic peptide substrate Boc-Gly-Pro-Arg-AMC. HAT was used as a positive control. As shown in Fig. 6A, high levels of enzymatic activity were measured on the surface of swAT-expressing cells, similar to the findings for HAT. In contrast, swTMPRSS2 seemed to possess low levels of proteolytic activity, if any, at the plasma membrane. To investigate whether enzymatically active swAT on the cell surface is able to activate the HA of incoming virus, MDCK cells transiently expressing protease were inoculated with A/Memphis (H1N1) possessing noncleaved HA0. Virions that contain HA0 are not able to infect cells unless the HA becomes cleaved at the stage of entry prior to fusion of viral and endosomal membranes at low pH. MDCK cells were transfected with plasmids encoding either swTMPRSS2, swAT, or HAT as a positive control. At 24 h p.t., cells were inoculated with A/Memphis (H1N1) virions containing HA0 and incubated for 10 h at 37°C to allow a single cycle of virus replication. Subsequently, infected cells were immunostained against NP and the number of infected cells was determined. Similar to HAT-expressing cells, cells expressing swAT could be efficiently infected with virus containing HA0 (Fig. 6B). Virus infection of cells was diminished in the presence of the protease inhibitor aprotinin, providing evidence for infection due to proteolytic activation of HA at the stage of entry. In contrast, swTMPRSS2-expressing cells failed to activate incoming virions containing noncleaved HA0. Finally, proteolytic activation of incoming virus was examined in primary PTECs and PBECs. Confluent PTEC and PBEC monolayers were inoculated with A/Memphis (H1N1) containing HA0 in the presence or absence of aprotinin, as described above. At 10 h p.i., infected cells were immunostained against NP and counted. As shown in Fig. 6C, cultures of primary PTECs and PBECs were infected with virus containing HA0, and infection was inhibited by aprotinin treatment. The data demonstrate that primary PTECs and PBECs support proteolytic activation of the HA of incoming virus, most likely due to enzymatically active swAT at the cell surface. In summary, our results demonstrate that swTMPRSS2 and swAT correspond to human HAT and TMPRSS2 not only in compartmentalization but also in respect to their enzymatic activity on the plasma membrane.

DISCUSSION

In the present study, we established cultures of primary porcine tracheal epithelial cells (PTECs) and porcine bronchial epithelial cells (PBECs) to study activation of influenza virus HA with a monobasic cleavage site by host proteases in the respiratory tract of pigs. Our data show that HA activation in the airways of swine may occur by similar proteases and at stages of the viral life cycle similar to those that have been described for the human airway epithelium.

Human influenza viruses of subtypes H1N1 and H3N2 replicated efficiently in both PTECs and PBECs, and virus containing cleaved HA was released from infected cells, providing evidence for expression of appropriate HA-activating proteases in these cells. Moreover, trypsin treatment only slightly increased the infectivity of released

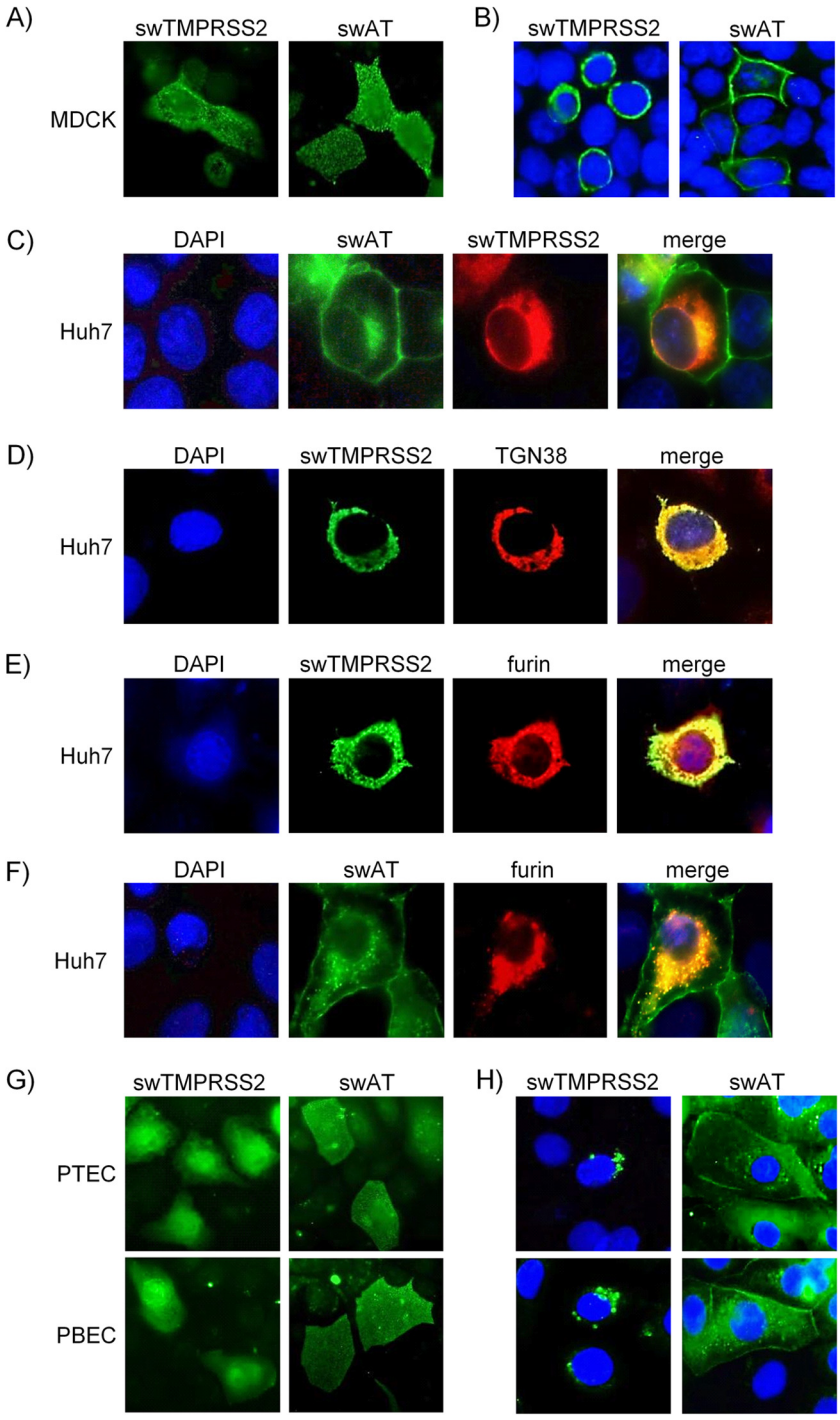

FIG 5 Localization of swTMPRSS2 and swAT in mammalian cells transiently expressing protease and primary PTECs and PBECs. (A) MDCK cells were transfected with either pCAGGS-swTMPRSS2-FLAG or pCAGGS-swAT-FLAG. At 24 h p.t., cell surface expression of swTMPRSS2 and swAT was analyzed using FLAG-specific antibodies and FITC-conjugated secondary antibodies. (B) MDCK cells transiently expressing swTMPRSS2 and swAT were fixed and permeabilized, and the intracellular localization of each protease was determined by indirect immunofluorescence using FLAG-specific antibodies and FITC-labeled secondary antibodies. Nuclei were counterstained with DAPI. (C) swTMPRSS2 and swAT transiently coexpressed in permeabilized Huh7 cells were visualized by polyclonal FLAG and HAT antisera, respectively, and fluorescence-labeled secondary antibodies. (D) Huh7 cells transiently expressing swTMPRSS2-FLAG were fixed, permeabilized, and analyzed for the localization of swTMPRSS2 and endogenous TGN38 using FLAG- and TGN38-specific primary antibodies and species-specific FITC- or TRITC-conjugated secondary antibodies. (E, F) Huh7 cells were cotransfected with plasmids encoding the TGN-residing protease furin and either swTMPRSS2-FLAG or swAT-FLAG for 24 h. The subcellular localization of the proteases was analyzed by FLAG- or furin-specific antibodies and species-specific fluorescence-conjugated secondary antibodies. (G) Cell surface expression of endogenous swTMPRSS2 and swAT in primary PTECs and PBECs was detected with polyclonal TMPRSS2 and HAT antisera and FITC-labeled secondary antibodies. (H) Monolayers of primary PTECs and PBECs were fixed and permeabilized, and endogenous swTMPRSS2 and swAT were visualized with anti-TMPRSS2 or anti-HAT serum, respectively, and FITC-conjugated antibodies.

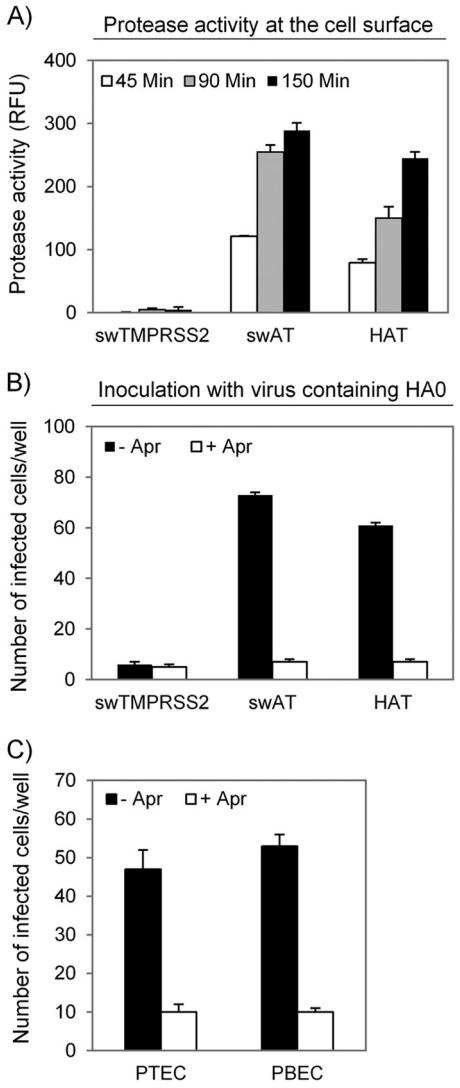

FIG 6 Enzymatic activity of swTMPRSS2 and swAT at the cell surface and proteolytic activation of incoming virions. (A) At 24 h after transfection of MDCK cells with either empty vector pCAGGS (mock) or expression plasmids encoding swTMPRSS2, swAT, or HAT as the control, the cells were incubated with the fluorogenic peptide substrate Boc-Gly-Pro-Arg-AMC. Protease activity at the cell surface was measured by determination of the relative fluorescence units (RFU) of the released AMC at the indicated time points. The results are the mean enzymatic activities for three independent experiments, with values for mock-transfected cells subtracted from values for protease-expressing cells within each experiment. (B, C) MDCK cells transiently expressing swTMPRSS2, swAT, and HAT (B) and primary PTEC and PBEC monolayers (C) were inoculated with human influenza virus isolate A/Memphis (H1N1) containing noncleaved HA0 ($\sim 10^3$ virions per well) and subsequently incubated for 10 h at 37°C to allow a single cycle of replication. Additional inoculation with virions in the presence of aprotinin (Apr) served as a control. Cells were fixed and immunostained against NP, and the number of infected cells per well was determined. The results are the mean values of a representative experiment, with each sample measured in triplicate.

virus, demonstrating that endogenous proteases support efficient HA activation in primary PTECs and PBECs.

RT-PCR analysis suggested that the proteases swAT and swTMPRSS2, which were assumed to be homologues of the human HA-activating proteases HAT and TMPRSS2, respectively, based on database analyses, are expressed in PTECs and PBECs. Cloning of swTMPRSS2 and swAT from primary PBECs and expression in mammalian cells demonstrated that both proteases cleave HA with a monobasic cleavage site, similar to trypsin, and support multicycle replication of H1N1 and H3N2 influenza A viruses. The protease swAT was cloned by using a predicted nucleotide sequence from porcine genome databases, and to our knowledge, swAT is described here for the first time. Cloning and expression of functional swTMPRSS2 and cleavage of HA upon coexpression in cell culture have been previously described by us and others (34, 35) and already indicated that the protease might support activation of influenza viruses.

RT-PCR analysis of swTMPRSS2- and swAT-specific mRNA suggests that both proteases are expressed in the epiglottis, trachea, and bronchi of pigs, whereas only swTMPRSS2 is present in porcine lung. Interestingly, the distribution of both proteases in porcine respiratory tissues corresponds to the expression of their homologues in the human airways. TMPRSS2 has been shown to be widely expressed in respiratory tissues, including the nose, larynx, trachea, bronchi, and lung (34, 36–38). Expression of HAT was reported to be prominent in the trachea and bronchi and has also been detected in epithelial cells of the nose, epiglottis, larynx, and esophagus, but it still remains unclear if HAT is present in the lung or not (34, 39, 40). In a recent study, human TMPRSS2 but not HAT was found to be expressed in myocytes, indicating that the protease may contribute to influenza-associated myocarditis (34). Expression of swAT and swTMPRSS2 in different porcine tissues, including the upper airways, the intestinal tract, and also myocytes, still remains to be investigated.

swAT and swTMPRSS2 turned out to be similar to HAT and TMPRSS2 also in respect to compartmentalization and enzymatic activity. Both proteases were found to be expressed on the cell surface, but only swAT was present as an active protease on the plasma membrane. According to this, swAT-expressing cells could be infected with virus containing HA0 due to proteolytic activation of HA on the cell surface upon entry. Moreover, the activity of swAT on the cell surface probably facilitated activation of incoming virus in primary PTECs and PBECs. Furthermore, swAT localized in vesicle-like structures in the cytoplasm of PTECs, PBECs, and transiently transfected Huh7 cells. However, thus far it is unknown whether swAT is already enzymatically active during intracellular trafficking or gains its enzymatic activity on the cell surface. Immunofluorescence microscopy of recombinant and endogenous swTMPRSS2 demonstrated that the protease accumulates within the cell and colocalizes with the TGN-residing proteins TGN38 and furin (29, 33). In general, TTSPs are predicted to be situated in the plasma membrane with an extracellular catalytic domain, and to our knowledge, this is the first report of the accumulation of a TTSP in the TGN. The missing enzymatic activity of surface-expressed swTMPRSS2 and its localization in the TGN strongly suggest that the protease cleaves HA in the TGN during its transport to the plasma membrane. Notably, highly pathogenic avian influenza viruses of subtypes H5 and H7 differ from other influenza viruses by possessing a multibasic HA cleavage site with the consensus sequence R-X-R/K-R. HA with such a cleavage motif is activated by the ubiquitous proteases furin and proprotein convertase 5/6 (PC5/6) in the TGN (41–43). In contrast, cleavage of HA by soluble proteases, such as trypsin, tryptase Clara, or the factor Xa-like protease, in embryonated chicken eggs (44) occurs outside the cell during and after the release of progeny virus. Therefore, cleavage of HA with a monoba-

sic cleavage site in general was assumed to occur extracellularly after virus release. The data presented here provide further support to the hypothesis that cleavage of HA with monobasic and multibasic cleavage sites can take place in the same compartment, the TGN, performed by different proteases (17, 45).

In this study, we focused on the identification and characterization of swine proteases homologous to HAT and TMPRSS2. However, additional HA-activating proteases might be present in the airways of swine. The TMPRSS2-related protease TMPRSS4 was shown to cleave influenza virus HA with a monobasic cleavage site *in vitro* (46). Furthermore, we demonstrated in a recent study that the TTSP matriptase, which is widely expressed in epithelial tissues in human and mouse (47, 48), is able to activate the HA of H9N2 influenza A viruses containing the di- or tribasic cleavage site motif R-S-S-R or R-S-R-R, in addition to HAT and TMPRSS2 (28). At present, it remains to be investigated if proteases homologous to matriptase or TMPRSS4 exist in swine. In previous studies, the trypsin-like proteases tryptase TC30 and mast cell tryptase (MCT) were purified from pig lungs and were shown to cleave HA with a monobasic cleavage site *in vitro* (49, 50). N-terminal protein sequencing revealed 70 to 80% homology of porcine mast cell tryptase and TC30 with mast cell tryptases from other species, but the genetic identity of both proteases is still unknown, and their contribution to influenza virus infection and spread *in vivo* remains to be investigated.

On the basis of their essential role for influenza virus infectivity and spread, HA-activating proteases represent potential drug targets. Early studies using the serine protease inhibitor aprotinin revealed that influenza virus replication in embryonated chicken eggs and mice can be markedly suppressed by inhibiting HA cleavage (51, 52). We further revealed that peptide-mimetic inhibitors of HAT and TMPRSS2 efficiently suppress influenza virus propagation in protease-expressing cell cultures (53–55). Remarkably, the combination of the protease inhibitor benzylsulfonyl-D-arginine-proline-4-amidinobenzylamide (BAPA) and the current NA inhibitor oseltamivir carboxylate was synergistic and blocked influenza virus propagation in human airway epithelial cells at concentrations lower than the concentration required by each inhibitor alone (53). Hence, inhibition of HA cleavage provides a promising approach for influenza treatment, and further development of the above-mentioned inhibitors may lead to novel influenza drugs.

In conclusion, we demonstrate that HA activation in the airways of swine and human shows a high similarity in respect to the relevant proteases, compartmentalization, and stages during the viral life cycle. For this reason, primary porcine respiratory epithelial cells provide valuable models to study HA-cleaving proteases and their inhibition. Interestingly, mice deficient in expression of TMPRSS2 and HAT lack a discernible phenotype (39, 56). Transgenic pigs with the knockout of either swTMPRSS2 or swAT may provide excellent animal models to confirm the role of either protease in HA activation *in vivo* and, moreover, to study the relevance of HA activation in terms of organ tropism, spread of infection, and virus pathogenicity.

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